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Sanjay Basnet

University of Nebraska - Lincoln, sbasnet2@unl.edu

Shripat T. Kamble

University of Nebraska - Lincoln, skamble1@unl.edu

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RNAi-Mediated Knockdown of *vATPase* Subunits Affects Survival and Reproduction of Bed Bugs (Hemiptera: Cimicidae)

Sanjay Basnet and Shripat T. Kamble

Department of Entomology, University of Nebraska–Lincoln,
103 Entomology Hall, Lincoln, NE

Corresponding author — S. Kamble, email skamble1@unl.edu

Abstract

The common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae) has resurged as one of the most troublesome household pests affecting people across the globe. Bed bug infestations have increased in recent years primarily due to the evolution of insecticide resistance and the insect's ability to hitchhike with travelers. *vATPases* are one of the most evolutionarily conserved holoenzymes in eukaryotes, which are mainly involved in proton transport across the plasma membranes and intracellular organelles. RNA interference (RNAi) has been developed as a promising tool for insect control. In this study, we used RNAi as an approach to knock down subunits A and E of the *vATPase* gene of bed bugs. Delivery of 0.2 μ g/insect of dsRNA specific to *vATPase-A* and *vATPase-E* into female bed bugs dramatically impaired the laying and viability of eggs over time. Injection of the *vATPase-E* dsRNA decreased survival of the bed bugs over 30 d. Our results also showed that the knockdown of mRNA is highly effective and persistent up to 30 d post injection. This research demonstrated that silencing of the two *vATPase* subunits A and E offers a potential strategy to suppress bed bug populations.

Keywords: RNA interference, *Cimex lectularius*, oviposition, egg hatching, survival

The common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae) is a hematophagous household pest which feeds primarily on humans. Increase in bed bug infestations across the world has significant health and economic impacts (Anderson and Leffler 2008, Doggett and Russell 2008). A bed bug bite can produce an allergic reaction in some individuals, with a conspicuous raised reddish rash that might itch for a few days (Ter Poorten and Prose 2005). Until now, there is no documented evidence of any human disease being transmitted by bed bugs. A recent laboratory study showed the *Trypanosoma cruzi*, a causative agent of Chagas disease, was transmitted in mice by bed bugs (Salazar et al. 2015). Therefore, the possibility of disease transmission in humans cannot be completely ignored. Pyrethroid and neonicotinoid insecticides are used for bed bug control, but evolution of resistance to these insecticides (Romero et al. 2007, Zhu et al. 2010, 2013, Tawatsin et al. 2011, Koganemaru et al. 2013, Dang et al. 2015) has made the control difficult. Other nonchemical approaches such as heat and ultracold treatments or biopesticides do not provide sustainable control.

Soon after the discovery of RNAi interference (RNAi) in the nematode *Caenorhabditis elegans* (Maupas) (Fire et al. 1998), the potential of RNAi to control several insects was established. The natural role of RNAi is to regulate gene expression and protect cells from viruses by turning off gene activity. Immediately after the introduction of dsRNA in a cell, the dsRNA is processed by Dicers or dicer-like helicases present in different organisms (Tijsterman and Plasterk 2004). The activity of Dicer mostly results in 21–23 long nucleotides called small interfering RNAs (siRNAs), and these siRNAs direct the RNA-induced silencing complex to the targeted mRNA and eventually degrades it (Hammond et al. 2000). This natural mechanism is being exploited to silence genes which have vital functions in insects by synthesizing dsRNA in vitro and delivering these to insects, leading to lethal phenotypes and reduction in growth and/or fecundity (Whyard et al. 2009, Huvenne and Smagghe 2010). The basic principle of this RNAi-based pest control strategy is the delivery of dsRNA to insects by mixing it in a diet, injecting a specific amount directly into the insect's hemocoel, spraying formulated concentrations directly on insects, or developing transgenic plants capable of producing dsRNA. However, the efficacy of the RNAi-based insecticides depends on the sensitivity of the target insect to RNAi. The RNAi systemic response (intercellular spreading of RNAi) varies among insects of different orders. *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) has a robust systemic RNAi, but a similar system has so far not been identified in *Drosophila melanogaster* Meigen (Tomoyasu et al. 2008). For *C. elegans*, *Sid-1* and *Sid-2* are involved in the uptake and spread of the RNAi across cells. Homologs of *Sid-1* are present in insects of different orders, such as Orthoptera, Phthiraptera, Hemiptera, Coleoptera, Lepidoptera, and Hymenoptera, but there is a high degree of variation in RNAi systemic response.

Insects of the Diptera lack the *Sid-1* homolog and do not exhibit systemic RNAi (Tomoyasu et al. 2008, Xu and Han 2008, Zotti and Smagghe 2015). Homolog of *Sid-1* has been reported for the hemipteran insect *Nilaparvata lugens* Stål (Zhang et al. 2013), consistent with a prominent systemic RNAi. Besides sensitivity to RNAi, other important parameters that determine viability of RNAi-based insect control methods include target gene selection, delivery of dsRNA under field conditions, and potential negative effects on nontarget organisms (Scott et al. 2013).

The vacuolar ATPases (*vATPases*) enzymes are a multisubunit complex with diverse functions in the eukaryotes (Nishi and Forgac 2002). Functionally, V1 and V0 are the two domains found in the *vATPases* enzyme, containing a total of 14 subunits and operate together as a rotatory machine (Forgac 2007). The peripheral V1 complex has eight different subunits (A–H) that carries out ATP hydrolysis, while the V0 is an integral membrane domain consisting of six subunits and directs translocation of protons (Nishi and Forgac 2002, Forgac 2007). Besides being integral to plasma membranes, *vATPases* are also present in the membranes of intracellular organelles, such as lysosomes, endosomes, vacuoles, and secretory vesicles. In insects, this enzyme is present in epithelial tissues of the Malpighian tubules, salivary glands, midguts, ovaries, and testis (Wieczorek et al. 1999, 2000). Because of the important biological roles they play in eukaryotic cells, *vATPases* have been studied as a potential RNAi target for insect control.

Based on literature search, previous RNAi studies on bed bugs are primarily focused on understanding the mechanisms of insecticide resistance (Zhu et al. 2012, 2013) or functions of genes (Gujar and Palli 2016a,b). In a recent study by Moriyama et al. (2016), the fecundity of female bed bugs was reduced after silencing the *vitellogenin* gene. The genome of bed bug has been published (Benoit et al. 2016), and this opens a door to screen potential RNAi target genes based on their roles in biological processes. In this study, we used the injection method to deliver dsRNAs specific to two subunits of *vATPases* and evaluated their potential to suppress the population of bed bugs. This study provides a new insight into developing a RNAi tool as a potential approach to controlling bed bugs.

Materials and Methods

Insect Colony

In this experiment, a Harlan strain of bed bugs was reared in glass jars (500 ml, VWR, International, Radnor, PA) containing Whatman filter papers (9 cm, Sigma-Aldrich, St. Louis, MO) as a substrate for oviposition. The glass jars were covered with organza fabric (JoAnn's Fabric, Lincoln, NE) to provide

ventilation. Bed bugs were maintained inside a growth chamber (Incubator I-35LL, Percival Scientific, IA) adjusted to a photoperiod of 14:10 (L:D), $25 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ RH (Montes et al. 2002). Expired reconstituted human blood containing red blood cells and plasma was obtained from the Nebraska Community Blood Bank (Lincoln, NE), warmed at $35\text{--}36^\circ\text{C}$, and fed to bed bugs through the Parafilm (Sigma-Aldrich) on a weekly basis.

Design and Synthesis of dsRNA

Total RNA was extracted from newly emerged adult bed bugs using a RNeasy Mini Kit (Qiagen, Valencia, CA), and $0.5\ \mu\text{g}$ of the total RNA was used to synthesize first strand cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). Sense and antisense primers were designed from mRNA sequences of *vATPase-A* (NCBI GenBank accession no.: LOC106673030) and *vATPase-E* (NCBI GenBank accession no.: LOC106667865) using the Primer3plus software (Untergasser et al. 2012). The primers were tailed with T7-promoter sequence (TAATACGACTCACTATAGGG) at the 5' region (Table 1) to initiate transcription of dsRNA from DNA templates. For dsRNA synthesis, the web-based program, siDirect was used to select a region in the mRNA sequence that potentially will generate the maximum number of siRNAs (Naito et al. 2004). Polymerase chain reaction (PCR) was performed using *Taq* PCR Core Kit (Qiagen), and the amplified product was purified with a QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol. The PCR products were sequenced at the DNA Sequencing Core facility (University of Nebraska, Omaha, NE) and confirmed by BLASTN. A $1.5\text{-}\mu\text{g}$ purified DNA was

Table 1. Primers for dsRNA synthesis and RT-qPCR

<i>dsRNA</i>	<i>Primer sequences for dsRNA synthesis</i>	<i>Base pair</i>			
<i>vATPase-E</i>	Forward: TAATACGACTCACTATAGGGGCGAGACTAAAAGCCCTCAA Reverse: TAATACGACTCACTATAGGGTTCCAAAGAGCGCATTTCTT	418			
<i>v-ATPase-A</i>	Forward: TAATACGACTCACTATAGGGTGTCTGGTGTATCACCTC Reverse: TAATACGACTCACTATAGGGTGGCAGAATCTGTCGTATG	382			
<i>GFP</i>	Forward: TAATACGACTCACTATAGGGGGTGATGCTACATACGGAAG Reverse: TAATACGACTCACTATAGGGTTGTTGTCTGCCGTGAT	370			
<i>RT-qPCR</i>	<i>Primer sequences for RT-qPCR</i>	<i>Base pair</i>	<i>Slope</i>	<i>R²</i>	<i>Efficiency</i>
<i>v-ATPase-E</i>	Forward: AGGTCGCCTTGTCCTCAAAAC Reverse: GCTTTTAGTCTCGCCTGGTTC	123	-3.28	0.99	101.8
<i>v-ATPase-A</i>	Forward: GATGGCCGATTCAACATCTC Reverse: GATGGCCGATTCAACATCTC	118	-3.29	0.99	101.45
<i>rpL8</i>	Forward: AGGCACGGTTACATCAAAGG Reverse: TCGGGAGCAATGAAGAGTTC	131	-3.40	0.99	98.6

used as a template for in vitro dsRNA synthesis in a 20- μ l reaction volume using a Megascript High Yield Transcription Kit (Ambion, Waltham, MA). The nontarget Green Florescence Protein (*GFP*) gene was used as negative control, which was prepared from pLZT/V5-His expression vector (Invitrogen, Waltham, MA) using gene-specific primers. The dsRNA was purified using a RNeasy Mini Kit (Qiagen) and eluted with ultrapure water. Later, the dsRNA quality was evaluated by gel electrophoresis and quantified using a NanoDrop ND-1000 spectrophotometry (Thermo Fisher Scientific, Waltham, MA).

Dose-Response of *vATPase-A* and *vATPase-E* dsRNAs

To test the optimum dsRNA dose that can cause the maximum knockdown of the targeted transcript, bed bugs were separately injected with 200 nl of one of four different doses 0.002, 0.02, 0.2, and 2 μ g/insect of dsRNA specific to subunits A and E of *vATPase*. The control group of insects were injected with 0.2 μ g *GFP* dsRNA/insect. Injection was performed on the ventral side behind the hind leg using a fine capillary tube (44 mm $\times$ 1.2 mm) fitted with a nanoinjector (Nanoject II Auto-Nanoliter Injector, Drummond Scientific Company, Broomall, PA). Four days after injection, three bed bugs per treatment were snapfrozen in liquid nitrogen and stored at -80°C until RNA isolation. cDNA was prepared from 0.5 μ g of the total RNA, and the transcript levels were measured by real-time quantitative PCR (RT-qPCR).

Bioassay With dsRNA Injections

Bed bugs used in this study were collected from three different generations maintained together in the growth chamber. Adult male and female bed bugs of 0–2 wk old were isolated and placed in glass jars for 2 d to allow mating. Males were sorted out and only females were injected with 200 nl of water or dsRNAs. Control groups included bed bugs injected with water, 0.2 μ g of *GFP* dsRNA/insect, and a noninjected group. Treatment groups included bed bugs injected with 0.2 μ g of dsRNA specific to *vATPase-A* and *vATPase-E*. Each treatment was replicated 10 times, each containing six females (two from each generation) confined in a Petri dish (50 mm $\times$ 11 mm). The Petri dish was provided with a sterile pad, which provides a suitable substrate for oviposition (Advantec MFS, Inc., Dublin, CA). After injection, the bed bugs were kept in a growth chamber for 12 h to recover and then provided with blood meal for 30 min in the dark. Only those bed bugs that fed to repletion were transferred to experimental units. Thereafter, bed bugs were fed every 10 d and transferred to the new Petri dishes. Petri dishes with eggs were kept inside growth chamber for 2 wk to allow sufficient hatching time. The average number of eggs laid by female bed bugs per feeding interval was calculated for 30 d. The average number of nymphs that emerged from the eggs were also assessed. In addition, the mean percent

survival of bed bugs was calculated. The eggs laid were observed under an Olympus SDF PIAPO 2× lens fitted to a stereomicroscope with an Olympus S2 16× lens (Olympus Imaging America Inc. Center Valley, PA). At day 3 and 30 post injection, three bed bugs per treatment were snap-frozen in liquid nitrogen and stored at -80°C for the gene knockdown study.

Real-Time Quantitative PCR

RT-qPCR was conducted using the Fast SYBR Green Master Mix (Applied Biosystems, Grand Island, NY) in a 7500 Fast System realtime PCR detection system (Applied Biosystems). Total RNA was extracted and 0.5 μg used to synthesize the first strand cDNA as described earlier. The cDNA used to determine primer efficiency was a five times dilution of the original concentration in the test reaction. The efficiency, slope, and amplicon length for RT-qPCR primers are provided (Table 1). The experimental set up for RT-qPCR included three biological and three technical replicates. The 10- μl reaction volume consisted of 2 μl of cDNA (dilution factor = 1: 20), 5 μl of SYBR Green, 0.4 μl of sense and antisense 5- μM primers, and 2.2 μl of water. The cycling parameters were 40 cycles each consisting of an initial holding at 95°C for 5 s and annealing at 58°C for 30 s followed by a melt curve analysis. A single melting curve ruled out primer dimer formation and non-specific product formations. Relative expression of transcripts was calculated using comparative $2^{-\Delta\Delta\text{Cq}}$ method (Livak and Schmittgen 2001). The mRNA levels were normalized using the internal control ribosomal protein L8 (*rpl8*) (Zhu et al. 2012).

Statistics

Experimental data on oviposition, hatching rate, and survival for every 10 d were analyzed separately as repeated measures analysis of variance (ANOVA). Repeated measures ANOVA can be used when the same parameters are measured at different time frames. Data on dose-response and gene knockdown were analyzed using one-way ANOVA ($P < 0.05$), and means were compared using Tukey Kramer's adjustment. All analyses were done with PROC GLIMMIX in SAS 9.4 (2013) (SAS Institute, Cary, NC).

Results

Effect of Different Doses of dsRNA on *vATPase* Knockdown

vATPase-A

All doses of *vATPase-A* dsRNA significantly reduced the mRNA levels compared to the *GFP* dsRNA ($F = 198.95$; $\text{df} = 4, 10$; $P < 0.0001$). The three doses

0.02, 0.2, and 2 $\mu\text{g}/\text{insect}$ were most effective in reducing the mRNA level, when compared to the dose 0.002 $\mu\text{g}/\text{insect}$ (Fig. 1A), suggesting doses above 0.002 μg are independent of the level of knockdown of the target.

vATPase-E

All doses of *vATPase-E* dsRNA significantly depleted the mRNA levels compared to that in insects injected with *GFP* dsRNA ($F = 81.09$; $df = 4, 10$; $P < 0.0001$), and no significant differences were observed among groups injected with different doses of *vATPase-E* dsRNA (Fig. 1B). Consistent with previous study with *vATPase-A*, the knockdown was dose-independent. For bioassays in subsequent experiments, we selected the dose 0.2 $\mu\text{g}/\text{insect}$.

RNAi Phenotypes

The only significant difference in survival of female bed bugs was observed 20–30 d following injection with *vATPase-E* dsRNA ($F = 36.58$; $df = 46.07, 4$;

Fig. 1. Dose-response of (A) *vATPase-A* dsRNA and (B) *vATPase-E* dsRNA in female bed bugs at day 4 after injection. Relative *vATPase-A* and *vATPase-E* transcript levels were normalized to *rpL8* and the relative quantification (RQ) values were analyzed. Means with different letters (a, b, and c) are significantly different. Means comparisons were performed using Tukey Kramer method (three biological and three technical replicates, $P < 0.05$).

$P < 0.0001$; Fig. 2). About 90% of insects treated with the *vATPase* dsRNA survived until the 20th day after injection, but this number decreased sharply to 18% thereafter. The survival of female bed bugs exposed to *vATPase-A* dsRNA showed a similar trend with the control groups. Although *vATPase-E* dsRNA appeared to produce more lethal effects on bed bugs in comparison to *vATPase-A* dsRNA, we found both dsRNAs had significantly affected oviposition ($F = 155.52$; $df = 45, 4$; $P < 0.0001$). The mean number of eggs laid by bed bugs injected with dsRNA specific to subunits A and E were less than three egg/female at the 0- to 10-d interval, 0.5 egg/female at the 10- to 20-d period, and no eggs were laid at the 20–30 d post injection period. Uninjected bed bugs laid significantly higher number of eggs than those injected with dsRNAs or water (Fig. 3A). The eggs laid by treated and untreated insects were also monitored for their ability to hatch. In contrast to the significant effect injection had on oviposition, there was no significant difference in the percentages of eggs hatched from eggs laid by the control insects, suggesting injection had no effect on egg hatching. The percentage of nymphs that emerged from eggs laid by bed bugs exposed to *vATPase-A* and *vATPase-E* dsRNA was significantly low than those of the control insects ($F = 2813.61$; $df = 45, 4$; $P < 0.001$). No significant difference in egg hatching was observed between the two treatment groups, *vATPase-A* and *vATPase-E* or among the three control groups (Fig. 3B). Most of the eggs laid by bed bugs exposed to *vATPase-A* and *vATPase-E* dsRNA were brownish with intact operculum, whereas those eggs laid by *GFP* dsRNA-injected bed bugs had a conspicuous opening from where the nymphs emerged (Fig. 4).

Fig. 2. Survival of female bed bugs ($n = 60$ /treatment) injected with *vATPase-A* and *vATPase-E* dsRNA, monitored at 10-d intervals and analyzed as repeated measures ANOVA. Means with different letters at each feeding interval are significantly different ($P < 0.05$).

Fig. 3. Effect of *vATPase-A* and *vATPase-E* RNAi in bed bug (A) oviposition and (B) egg hatching. Data analyzed as repeated measures ANOVA. Control groups: water, *GFP* dsRNA (0.2 μ g), and uninjected, Treatment groups: *vATPase-A* (0.2 μ g) and *vATPase-E* (0.2 μ g) dsRNA. Means with different letters at each feeding interval are significantly different ($P < 0.05$).

Fig. 4. Phenotypes of eggs laid by bed bug injected with *vATPase-A* and *vATPase-E* dsRNA. Bed bugs were injected with 0.2 μ g of dsRNA specific to *GFP* (control), *vATPase-A*, or *vATPase-E*. The images were photographed using Olympus SDF PIAP0 2 $\times$ lens fitted to stereomicroscope with Olympus S2 16 $\times$ lens.

RT-qPCR Analysis

The transcript levels of the targeted genes were assessed at 3 and 30 d after dsRNA injection. Gene expression data analyzed from bed bugs injected with *vATPase-A* dsRNA showed significant reduction of targeted mRNA levels at 3 d ($F = 10.81$; $df = 2, 6$; $P < 0.05$; Fig. 5A) and 30 d ($F = 36.26$; $df = 2, 6$; $P < 0.05$, Fig. 5B). Similarly, the mRNA from bed bugs injected with *vATPase-E* dsRNA were significantly depleted at 3 d ($F = 22.97$; $df = 2, 6$; $P < 0.05$; Fig. 5C) and 30 d after dsRNA injection ($F = 34.91$; $df = 2, 6$; $P < 0.05$; Fig. 5D). We also assessed whether the knockdown of subunit A had a compensatory effect on the mRNA level of subunit E and vice versa. Knockdown of subunit A had no significant effect on the abundance of mRNA level of subunit E ($F = 2.61$; $df = 2, 6$; $P < 0.15$; Fig. 6A). In contrast, the bed bugs injected with *vATPase-E* dsRNA had a significant increase in the abundance of subunit A mRNA ($F = 11.89$; $df = 2, 6$; $P < 0.05$; Fig. 6B).

Fig. 5. Relative *vATPase-A* transcript abundance in female bed bugs at **(A)** day 3, and **(B)** day 30 post injection. Relative *vATPase-E* transcript expression in female bed bugs at **(C)** day 3, and **(D)** day 30. Relative *vATPase-A* and *vATPase-E* transcript levels were normalized to *rpl8* and the relative quantification (RQ) values were analyzed. Means with different letters (a, b) are significantly different. Means comparisons were performed using Tukey Kramer method (three biological and three technical replicates, $P < 0.05$).

Fig. 6. Transcript level of subunit E after injection with **(A)** *vATPase-A* dsRNA, and abundance of subunit A mRNA after injection with **(B)** *vATPase-E* dsRNA into female bed bugs at day 30 post injection. Relative transcript levels were normalized to *rpL8* and the relative quantification (RQ) values were analyzed. Means comparisons were performed using Tukey Kramer method (three biological and three technical replicates, $P < 0.05$).

Discussion

This study demonstrates that injection of dsRNA specific to the *vATPase* gene affects the behavior, survival, and reproduction of bed bugs. Less than 20% of the treated female bed bugs survived over a 30-d period. Lethality was observed only from the injection of the dsRNA of subunit E, which suggests that the subunit plays an important role in the development of bed bugs. The subunit A is the catalytic site of the holoenzyme, while the subunit E crosslinks with several other subunits such as the B, G, and C to form the peripheral stalk of the proton pump (Nishi and Forgac 2002). Knock-down of subunit E may have induced a compensatory expression of subunit A which could have been lethal to the bed bugs. Mortality has been observed by the knockdown of subunit E in *D. melanogaster*, *Manduca sexta*

L. (Lepidoptera: Sphingidae), *T. castaneum*, and *Acyrtosiphon pisum* Harris (Hemiptera: Aphididae) (Whyard et al. 2009). Knockdown of other subunits has also produced lethal phenotype in other insects. For example, knockdown of subunit A was lethal in *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (Baum et al. 2007, Rangasamy and Siegfried 2012), and *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Thakur et al. 2014), as was subunit B in *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) (Badillo-Vargas et al. 2015), and *Peregrinus maidis* Ashmead (Hemiptera: Delphacidae) (Yao et al. 2013), and subunit D in *P. maidis* (Yao et al. 2013). Our data on dsRNA dose-response demonstrated significant knockdown of targeted transcript with all the doses tested, which suggests that a relatively low dose of dsRNA was enough to knock down the transcripts significantly. We also observed significant knockdown of *vATPase-A* and *vATPase-E* at day 30 after injections, indicating persistence of RNAi in the bed bugs for at least a month.

Our data on oviposition and egg hatching further sheds light on high reproductive capacity of bed bugs when fed regularly. These findings are consistent with the fact that bed bugs are prolific reproducers (Polanco et al. 2011). A new generation can be established in 1–2 mo, with the possibility of multiple generations within a year. This high fecundity is one of the reasons for the increase in bed bug infestations. Our results demonstrated that RNAi of the two subunits of *vATPase* has great potential to reduce fecundity of bed bugs. Most eggs laid by the bed bugs injected with *vATPase-A* and *vATPase-E* dsRNAs were brownish, which implies necrosis of the embryo. The disruption in proper embryogenesis probably led to the observed low percentage of eggs laid by dsRNA-treated females. The *vATPase* holoenzyme is reported to be abundantly present in the cell membrane of ovarian follicles and vesicles and is actively involved in ion transport, fluid absorption (Wieczorek et al. 2000, 2009), and yolk processing necessary for follicle growth (Bohrmann and Braun 1999). Lack of ion transport to the ovarian follicles could be the primary cause of the sharp decline in the egg hatching rate. *vATPase* also has an important function in the male reproduction process, which involves sperm maturity and mobility across epididymis and vas deferens in vertebrates (Brown and Breton 2000). It would be interesting to study the effect of *vATPase* RNAi on sperm mobility and fertility in the future.

In addition to previous several reports (Siomi and Siomi 2009), our results indicated that injections can be successfully used to deliver dsRNA into bed bugs in laboratory conditions. However, dsRNA delivery into bed bugs in field conditions for the purpose of pest management is somewhat challenging. Our unpublished data have shown that dsRNA is unstable in blood, and therefore, feeding dsRNA to bed bugs by mixing with blood does not seem feasible in this case. More research is needed to develop an innovative

dsRNA delivery technique for hematophagous insects. Unlike hematophagous insects, phytophagous insects can be exposed to dsRNA produced by transgenic plants. Miguel and Scott (2016) showed that foliar application of dsRNA on potato leaves effectively controlled Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), suggesting that foliar application of dsRNA can be effective in some insects. However, further detailed research is necessary to evaluate that the dsRNA can effectively penetrate the cuticle of bed bugs after contact. Most importantly, off target effects of RNAi insecticides to mammals and beneficial insects should be studied extensively. Specificity of RNAi insecticides can be enhanced by selecting the regions of genes that do not match sequences of the mammalian genome or targeting genes that are not well conserved in mammals (Scott et al. 2013).

In conclusion, RNAi-mediated knockdown of two subunits A and E of *vATPase* inhibited reproduction and specifically knockdown of subunit E produced lethal phenotype in female bed bugs. This research demonstrates the potential of RNAi as a strategy to suppress bed bug populations and provides foundation for developing RNAi-based insecticides for bed bug control.

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